AD 407 380

(5) NA 63-4-1

To: European Research Office (9851 DU)
U.S. Department of the Army,
FRANKFURT/Main, Germany.
A.P.O. 757, U.S. Forces.

Subject of the research:

Advanced studies on the effect of ultraviolet light on certain constituents of nucleic acids and their interaction with adjacent water molecules under these conditions.

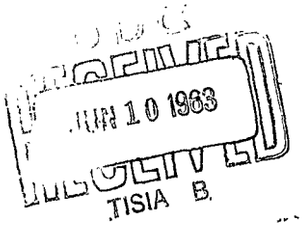
Name of Contractor: Prof. W. Berends,
Director Biochemical and Biophysical Laboratory of the Technological University,
Julianalaan 67,
Delft.
The Netherlands.

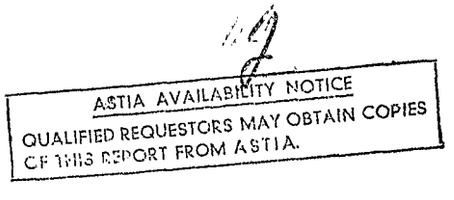
Contractnumber: DA - 91 - 591 - EUC - 2022.

Type and number of report: Final Technical Report.

Period covered by the report: 1 November 1961 - 1 November 1962.

The research reported in this document has been made possible through the support and sponsorship of the U.S. Department of Army, through its European Research Office.





In continuation of our research on the U.V. sensitivity of nucleic acids we have been engaged in the study of the effect of this kind of radiation on biologically active nucleic acids. As a logical consequence of this study and the results obtained in an investigation of energy transfer in aqueous solutions, we have extended our program to the photosensibilization of nucleic acids and related substances by dyes. Though the effect of U.V. or visible light in the presence of certain dyes may both lead to inactivation of nucleic acids, the chemical background appears to be different.

In connection with this work several other problems had to be investigated. The isolation of the so-called Kornberg enzym (D.N.A. polymerase) had to be done in order to be able to prepare model compounds like A-T polymers. These polynucleotides are wanted for a study of the effect of U.V. on polymers of known composition. Apart from this further attention was paid to the effect of visible light on several dyes and the energy transfer of excited molecules in aqueous solution.

The results obtained so far will be discussed in this report.

I. Effect of U.V.-light on the biological activity of special nucleic acids.

Our initial studies on the effect of U.V. on nucleic acids were performed with biologically inactive, commercial products. The study of model compounds, like the nucleotides and the bases occurring in the nucleic acids, has led us to the discovery of the photodimerization of thymine.[1, 2, 3, 4]

---

1. A. Rörsch, R. Beukers, J. IJlstra and W. Berends, Rec.trav.chim., 77 (1958) 423.
2. R. Beukers, J. IJlstra and W. Berends, Rec.trav.chim., 77 (1958) 729; 78 (1959) 247, 879, 883; 79 (1960) 101.
3. R. Beukers and W. Berends, Biochim.Biophys.Acta, 38 (1960) 573; 41 (1960) 550; 49 (1961) 181.
4. W. Berends, J.Chimie Physique 1961, 1034.

It is generally accepted at present that this dimerization is the main reaction as the result of U.V.-irradiation of nucleic acids.[5] Nevertheless it must be emphasized that a relatively rather high percentage of dimerization must occur before it can be detected analytically. . The question has still to be answered whether the high sensitivity of biologically active nucleic acids to U.V. is also due to dimerization. For this reason we wanted to study the chemical alterations that lead to the inactivation of 1. a transforming principle of Bacillus subtilis (a deoxyribonucleic acid preparation) and 2. the so called soluble ribonucleic acid of rat liver.

1. Small changes in the structure of the transforming deoxyribonucleic acid are detectable by a decrease in the ability of the preparation to transfer a special genetic factor to the acceptor strain. The effect of U.V. light on transforming factor has been studued already by a number of investigators (for a survey: J.H. Stuy)[6]. On the basis of our work the inactivating effect of this radiation is supposed to be due to the conversion of thymine molecules in thymine dimers and other photoproducts. We noticed already in earlier investigations that not all of the thymine converted is found back as thyminedimer.[3] This has been confirmed by others[5] and we decided to study this phenomenon with a deoxyribonucleic acid preparation containing ^{14}C labeled thymine.

Incubation of a wild-type strain of Bacillus subtilis in a medium containing 2-^{14}C uracil leads to the formation of DNA with 2-^{14}C thymine and 2-^{14}C cytosine. The DNA was isolated according to Marmur.[7] A sample containing 1 mg DNA (about 5 ml) was irradiated with a General Electric Germicidal lamp (4 W, mainly 253.7 mμ radiation) during 15 minutes at a distance of 5 cm.

---

5. e.g. K.C. Smith in A.C. Giese (ed.), "Photobiology: Action of light on living materials" (to be published).

6. J.H. Stuy, "Radiation inactivation of intracellular transforming deoxynucleic acid", Thesis 1961, Utrecht.

7. J. Marmur, J.Mol.Biol. 3 (1961) 208.

The mixture was lyophilized and subsequently hydrolysed with 10 N perchloric acid for 1 hour at 100° C to obtain the free purines and pyrimidines. Finally these substances were separated by paperchromatography (solvent butanol-NH_3)[8].

With a gas-flow detector (Tracerlab) the paper was scanned and the radioactivity found was plotted on graph paper. (fig. 1)

The percentage of thymine in the irradiated sample was about 30% less than in the control. One third of the amount disappeared was converted into thymine-dimer, while the rest of the radioactivity remained at the starting line of the chromatogram and at an R_f 0.1. The percentage of cytosine appeared unchanged. Nothing is known about the nature of the photoproducts formed. Dr K.C. Smith (Stanford University) has obtained the same results and is presently engaged in the isolation and identification of the other photoproducts of thymine.[5]

2. The soluble ribonucleic acids of the living cell bind the activated amino acids and transfer these to the ribosomes where they are incorporated in newly formed protein. Therefore we have two ways of testing the activity of a soluble ribonucleic acid. First the ability to bind an amino acid and in the second place the efficiency of the amino acid incorporation into protein. In preliminary experiments we have been able to detect a decrease of the activity after U.V. irradiation of the ribonucleic acid, sRNA isolated from rat liver. The preparation of this nucleic acid and of the enzymes needed for the testing of the sRNA was carried out according to Hoagland et al.[9]

Fresh liver is cooled in a buffer solution (0.02 M phosphate buffer pH 7.8, 0.025 M KCl, 0.05 M Tris buffer p_H 7.6, 0.125 M sucrose, 0.01 M nicotinamide and 0.005 M $MgCl_2$) and homogenated (Potter). The cell debris was removed by centrifugation at 20.000 g for 10 minutes and the ribosomes by centrifugation at 105.000 g for 2 hours. The supernatant obtained is called

---

8. W.S. Mac Nutt, Biochem.J. 56 (1952) 384.

9. M.B. Hoagland, M.L. Stephenson, J.F. Scott, L.I. Hecht, P.C. Zamecnik, J.Biol.Chem. 231 (1958) 241.

105,000 g fraction and contains the enzymes necessary for activation of the amino acids and binding these to the sRNA. This fraction also contains a certain amount of sRNA and is therefore used as a source of this material. The isolation normally consists of shaking the 105,000 g fraction with an equal volume of a 90% phenol solution in water (10 minutes) and a precipitation of the nucleic acid from the aqueous layer by ethanol (endconcentration 67%). The preparation is purified by passing it over a column of Sephadex G 25.

To test the amino acid binding capacity of the sRNA obtained about 0.1 mg is incubated with 0.1 ml 0.01 M ATP, $\frac{1}{2}$ ml of the 105,000 g fraction and s sufficient amount of ^{14}C labeled amino acid at 37° C for 20 minutes. To remove the radioactivity not incorporated the protein and the sRNA is precipitated at 4° C with perchloric acid 10% and thoroughly washed with diluted perchloric acid (0.2 M). A certain part of the protein, the polysaccharides and the lipids are removed by subsequent washing with perchloric acid-ethanol (1:5), ethanol, ethanol-ether and ether. The radioactivity of the residue is determined with a ^{14}C counting device, in this case a Tracerlab gas flow counter and scaler. Some representative figures are given below for the inactivation of sRNA by an U.V. dosis of 10^6 ergs.

Not irradiated 85 cpm, irradiated 50 cpm. Inactivation 45%. This inactivation proved to be partially due to a heat reactivable hydration of uracil and/of cytosine according to Sinsheimer.[10] Warming of the sRNA solution at 60° C for about 30 minutes immediately after the irradiation namely results in a partial recovery of the activity: Not irradiated, warmed: 87 cpm, irradiated, warmed 68 cpm. Inactivation 22%. It will be necessary to study this effect in more detail. From the results ohtained so far one has to conclude that at least part of the inactivation is due to a hydration of the pyrimidines. We expect however that uracil dimerization also takes place.

---

10. R.L. Sinsheimer, Rad.Res. 1 (1954) 505; 6 (1957) 121.

II. Photosensitization of nucleic acids.

We may divide our research of the photosensitization in two separate studies. The first is a direct investigation of the effect of visible light in the presence of a dye on a nucleic acid preparation. The second consists in a more theoretical consideration of the way of energy transfer from the dye to the sensitive substance. This study has mainly been concerned with very special model systems.

1. Direct photosensitization of deoxyribonucleic acid.

It is possible to inactivate deoxyribonucleic acid with visible light in the presence of dye just as it is possible to inactivate with U.V. The nucleic acid itself is not influenced by the light on account of the lack of absorption in this part of the spectrum (400-800 mμ).

When a dye is added tot the solution the possibility exists that this dye absorbs part of the light and transfers the energy in one way or another to the deoxyribonucleic acid, causing chemical changes in this polymer. The dye, having this property, is called photosensitizator while the whole phenomenon is known as photosensitization or photodynamic action.

A transforming principle is a very well suited object for a study of the photosensitization of a deoxyribonucleic acid. The molecular changes brought about by this photosensitized inactivation cannot be determined by direct spectrophotometry.

The effect of visible light plus dye on the biological activity of a transforming factor was already studied.[11]

We decided to investigate the photosensitization on a molecular level hoping to detect a simple change in the nucleic acid just as the photodimerization of thymine in DNA upon U.V. irradiation. We have chosen the Bacillus subtilis transforming system as a testing object, The transforming experiments were carried out according to Anagnostopoulos and Spizizen.[12]

---

11. J.S. Bellin, G. Oster, Bioch.Biophys.Acta, 42 (1962) 533.
12. C. Anagnostopoulos, J. Spizizen, J.Bact. 81 (1961) 741.

It proved to be possible to label the DNA of B-subtilis just by adding a radioactive pyrimidine or purine (20 μC/liter) to the normal medium. Addition of 2-^{14}C uracil leads to a DNA-preparation containing 2-^{14}C cytosine and 2-^{14}C thymine; upon adding 8 ^{14}C adenine, a DNA was obtained with 8 ^{14}C guanine and 8 ^{14}C adenine. With these DNA's it is possible to detect small changes, i.e. disruption of one of the bases by lyophilizing the solution after the irradiation and subjecting it to hydrolysis by 10 N perchloric acid for 1 hour at 100° C. The pyrimidines and purines were separated by paperchromatography and the radio-activity was measured as described above (p. 3).

The following dyes have been investigated in their ability to inactivate biologically active deoxyribonucleic acid photo-dynamically.

1. berberine
2. neutral red
3. methylene blue
4. fluorescein.

To test the photodynamic activity of the dyes 2 ml of a transforming DNA solution (0,2 mg/ml) was mixed with 2 ml of a 10^{-5} M solution of the dye in water. The sample was illuminated in a cuvet with two Philips HP 125 high pressure mercury lamps (one on each side, the light was filtered by a 2½ cm layer of water to prevent the solution from warming up) during 45 minutes at a distance of 20 cm. The transforming activity of the DNA irradiated was tested according to Spizizen.[12] This was done in the presence of the dye. The controls consisted of transforming DNA with dye kept in the dark.

Table I.

Activity of DNA after irradiation with visible light in the presence of dye

Unirradiated DNA	alone	set to 100
DNA irradiated	without dye	100
DNA + neutral red	unirradiated	110
DNA + neutral red	irradiated	50
DNA + methylene blue	unirradiated	97
DNA + methylene blue	irradiated	90
DNA + fluorescein	unirradiated	110
DNA + fluorescein	irradiated	65

In the presence of berberine the growth of Bacillus subtilis is stopped. It was therefore not possible to test this dye with regard to its photodynamic action.

The reaction in the presence of neutral red, the most active dye in this system, has been studied in more detail. It was observed by others that an oxydation of guanine takes place during the illumination of DNA in the presence of methylene blue.[12a] Assuming a similar action of neutral red, a deoxyguanylic acid-neutral red system was studied with a Bausch and Lomb spectronic 505 automatic spectrophotometer. The mixture to be irradiated consisted of 0,5 ml deoxyguanylic acid (2.10^{-4} M), 0,5 ml neutral red solution (2.10^{-4} M) and 4 ml buffer solution (pH=6). Time of irradiation one hour, distance 20 cm, source, Philips HP 125. Neutral red itself however is not stable under these conditions and the decrease in extinction obtained for the deoxyguanylic acid-neutral red solution was diminished by that of the neutral red solution alone. It was possible to show a conversion of 5-10% of the nucleotide. No such effect was obtained with the other nucleotides studied, deoxycytidylic acid, thymidylic acid and deoxyadenylic acid.

Subsequently deoxyribonucleic acid containing 8 ^{14}C adenine and 8 ^{14}C guanine was isolated from Bacillus subtilis according to Spizizen[12] by growing the bacteria in the presence of 8 ^{14}C adenine (20 µC/liter medium). A mixture of 1 ml DNA solution (0,2 mg) and 2,5 ml neutral red solution (4.10^{-5} M) was irradiated during 20 hours with a Philips HP 125 lamp. In the control experiment DNA was irradiated in the absence of dye. The solution was lyophilized and the residue hydrolyzed to the bases by 10 N perchloric acid (1 hour 100° C) and finally the bases were separated by paperchromatography and detected and measured as described above. It was found that this irradiation resulted in a 16% decrease of the guanine content.

The radioactivity of the guanine, that had disappeared was partly found to escape as $^{14}CO_2$ during the hydrolysis with perchloric acid. This was demonstrated by leading the gaseous products into a bariumhydroxyde solution and measuring the

12a. M.I. Simon, H. van Vunakis, J.Mol.Biol. 4 (1962) 488.

radioactivity of the precipitate formed. Consequently the initial photoproduct is further broken down during the hydrolysis. If the irradiation was performed in a nitrogen atmosphere, no decrease of the guanine content was observed. From these results it seems very likely that an oxydation is responsible for the inactivation phenomenon.

A complicating factor in using neutral red or methylene blue is the rapid conversion of these dyes by the light used. For this reason we have been looking for a more stable dye with the same sensitizating properties. Oster[11] found riboflavin to be active as well and since in our researchprogram lumichrome has been used as a stable substituent of riboflavin we decided to test this component in our system.

In the presence of lumichrome, deoxyguanylic acid appeared to be broken down very fast upon irradiation by visible light (for experimental details see figure 2). The course of the lumichrome photosensitized reaction of deoxyguanylic acid in dependence of time was studied spectrophotometrically (Bausch & Lomb Spectronic 505). The spectrum of the guanylic acid changed through two isosbestic points (figure 2). This means that a stable product is formed during the irradiation. Again no reaction was found to take place in a nitrogen atmosphere so an oxydation is likely. The same phenomenon was observed with riboguanylic acid and guanine. Guanine, however, showed a more complicated course of the reaction. During the first minutes of irradiation a reaction comparable with that of guanylic acid takes place. That means that exactly the same isosbestic points are found. By irradiation over longer periods, however, a spectrum deviating from these points was obtained. The p_H of the solution apparently determines the course of the reaction. It looks as if in the case of the nucleotides the first photoproduct is protected against further reactions by the sugar-phosphate or by the substitution of the nitrogen at the 9 position. Experiments with deoxyribonucleic acid containing ^{14}C guanine proved that irradiation with visible light in the presence of lumichrome also leads to a conversion of the guanine. It is possible to convert at least 80% of the guanine in this way. The reaction looks very similar to

that with neutral red: again the formation of $^{14}CO_2$ could be demonstrated to occur during the hydrolysis with perchloric acid.

The photoproduct of guanylic acid apparently being rather stable justified an attempt of isolation and identification. The solution becomes slightly yellow on irradiation. The reaction mixture was subjected to paperchromatography and thin-layer chromatography in order to try to isolate the photoproduct. The chromatography on paper was carried out as described above. The thin layer chromatography was performed according to Randerath[13], solvent t.amylalcohol-formic acid-water 3:2:1. A newly formed substance was found by way of its yellow colour, its U.V. absorption and its positive phosphate test.[14] This substance could be isolated in a very small amount. The absorption-spectrum looks very much the same as that of the irradiation products of guanylic acid and guanine. To obtain more of the photoproduct a separation of the mixture on a larger scale is necessary. The irradiated solution contains lumichrome, one or more photoproducts and the non-converted guanylic acid. A separation is possible with column chromatography. With cellulose, however, we ran into difficulties, on account of the instability of the photoproduct in acid solvents and the high degree of dilution during the elution (so hampering the detection). The same disadvantage has a Dowex-1 ion exchange column. Moreover the product is very strongly absorbed to this material. At the moment experiments with a triethylaminoethyl cellulose column look very promising.[15]

The irradiation of deoxyguanylic acid in the presence of lumichrome has also been studied in a Warburg apparatus (Hormuth) in order to determine the amount of oxygen needed for the reaction. Irradiation was performed by direct subjecting the contents in the Warburg flasks to the light of a Philips HP 125 lamp. The irradiated mixture was 3 µM deoxyguanylic acid and 0.07 µM lumichrome in 2 ml water. The oxygen consumption is 1.2 mol. per mol. nucleotide. To our surprise CO_2 is formed during the illumination

---

13. K. Randerath, Bioch.Bioph.Res.Comm. 6 (1962) 452.

14. Procedure described by H.E. Wade and D.M. Morgan, Nature 171 (1953) 529.

15. see C.L. Davey, Bioch.Bioph.Acta, 61 (1962) 538.

(0.8 mol. per mol. nucleotide). Further experiments revealed that the irradiation of deoxyribonucleic acid (containing ^{14}C guanine) also leads to the formation of $^{14}CO_2$. We hope to find a solution for this behaviour by the identification of the yellow photoproduct.

Our work with lumichrome has led us to the conclusion that the transfer of energy to stilbene derivatives occurs on a triplet level. To check this for the reaction first mentioned we have studied the influence of paramagnetic and diamagnetic metal ions on the conversion of deoxyguanylic acid. If we are dealing with a triplet-triplet transfer paramagnetic ions will slow down the reaction. These ions namely shorten the time of existence of the excited molecules in the triplet state, thereby diminishing the chance that a reaction by collision takes place. A mixture of 3.10^{-2} µM lumichrome, 25.10^{-2} µM deoxyguanylic acid and 3.10^{-3} M of the metal ion, total volume 5 ml, was irradiated with two Philips HP 125 lamps at 20 cm distance during 5'. The changes in the spectrum were recorded with the Bausch and Lomb spectronic 505. It was found that the paramagnetic metal ions, Co$^{\cdot\cdot}$, Ni$^{\cdot\cdot}$, Mn$^{\cdot\cdot}$ and Cu$^{\cdot\cdot}$ do slow down the conversion of deoxyguanylic acid, while no effect is observed on adding diamagnetic ions like Cd$^{\cdot\cdot}$, Ca$^{\cdot\cdot}$, K$^{\cdot}$ or Zn$^{\cdot\cdot}$.

Table II.

Added metal	% inhibition
Co$^{\cdot\cdot}$ ions	55
Mn$^{\cdot\cdot}$ ions	45
Cu$^{\cdot\cdot}$ ions	90
Ni$^{\cdot\cdot}$ ions	55
Cd$^{\cdot\cdot}$ ions	no
Ca$^{\cdot\cdot}$ ions	no
K$^{\cdot\cdot}$ ions	no
Zn$^{\cdot\cdot}$ ions	no

The addition of Zn$^{\cdot\cdot}$ causes a change in the absorption-spectrum between 300 and 380 mµ, but this is probably due to the formation of a Zn-complex of the photoproduct. One would expect the paramagnetic oxygen molecules to have the same effect as the metal ions. However, oxygen is essential for the reaction and it is therefore impossible to remove this gas.

Less advanced than the work with deoxyribonucleic acid has been the study of the photozensitization of soluble ribonucleic acid. In collaboratorium with Dr L. Bosch from the State University of Leiden the influence of a number of dyes on the activity of this nucleic acid was tested in the same way as described before (pag. 4).

The irradiation procedure consists in illuminating the sRNA in the presence of the dye under investigation (at a concentration of 10^{-5} M) with the light of a high pressure mercury arc, filtered by a glass filter to remove the shorter wavelengths. After completion of the irradiation the dye was removed by passing the mixture over a Sephadex G 25 column. The dye is absorbed while the sRNA passes unhampered. The solution obtained is concentrated by lyophilization. The controls consisted of the same solutions handled in the same way except for the irradiation. The filtration and lyophilization did not have any influence on the activity of the sRNA to bind amino acids. Under the conditions used neutral red and 8-methoxypsoralen have no effect on the activity. Berberine, however, causes a 30% decrease in activity.

0.3 mg sRNA, irradiated without dye 180 cpm
unirradiated 190 cpm

0.3 mg sRNA, irradiated with neutralred
200 cpm
unirradiated 210 cpm

0.3 mg sRNA, irradiated with 8 methoxypsoralen
200 cpm
unirradiated 195 cpm

0.3 mg sRNA, irradiated with berberine
140 cpm
unirradiated 200 cpm.

It is too early yet to claim a specific action of berberine. We are presently involved in testing the influence of these dyes on the incorporation of the labelled amino acids into the microsome fraction. All these experiments have been carried out with enzymes and sRNA obtained from rat liver. We have started a similar study with the same system from yeast and we are planning to include experiments with bacteria to check the universality of the effects obtained.

Less advanced than the work with deoxyribonucleic acid has been the study of the photozensitization of soluble ribonucleic acid. In collaboratorium with Dr L. Bosch from the State University of Leiden the influence of a number of dyes on the activity of this nucleic acid was tested in the same way as described before (pag. 4).

The irradiation procedure consists in illuminating the sRNA in the presence of the dye under investigation (at a concentration of 10^{-5} M) with the light of a high pressure mercury arc, filtered by a glass filter to remove the shorter wavelengths. After completion of the irradiation the dye was removed by passing the mixture over a Sephadex G 25 column. The dye is absorbed while the sRNA passes unhampered. The solution obtained is concentrated by lyophilization. The controls consisted of the same solutions handled in the same way except for the irradiation. The filtration and lyophilization did not have any influence on the activity of the sRNA to bind amino acids. Under the conditions used neutral red and 8-methoxypsoralen have no effect on the activity. Berberine, however, causes a 30% decrease in activity.

0.3 mg sRNA, irradiated without dye 180 cpm
unirradiated 190 cpm

0.3 mg sRNA, irradiated with neutralred
200 cpm
unirradiated 210 cpm

0.3 mg sRNA, irradiated with 8 methoxypsoralen
200 cpm
unirradiated 195 cpm

0.3 mg sRNA, irradiated with berberine
140 cpm
unirradiated 200 cpm.

It is too early yet to claim a specific action of berberine. We are presently involved in testing the influence of these dyes on the incorporation of the labelled amino acids into the microsome fraction. All these experiments have been carried out with enzymes and sRNA obtained from rat liver. We have started a similar study with the same system from yeast and we are planning to include experiments with bacteria to check the universality of the effects obtained.

2. Energy transfer in aqueous solution.

During work on the mechanism of action of the fungicide pimaricin on yeast cells it was observed that visible light destroys the pimaricin if riboflavin is present.[15a] This effect is remarkable because pimaricin does not show any absorption in the visible region. Since the presence of riboflavin is obligatory for the destruction of the pimaricin, it appears that the riboflavin absorbs the light and brings about the destruction of pimaricin. Pimaricin belongs to a group of polyene antibiotics.[16-18] Though the structure of the molecule is rather complicated, the part of the molecule which is responsible for the ultraviolet spectrum is practically limited to a tetraën grouping. Compounds with a reactive system of conjugated double bonds are usually very sensitive to free radicals, which could have their origin in the decomposition of riboflavin by light.[15a]

Though the molecule of pimaricin is very complex, we have initially continued to use this compound since a change under the influence of light can be detected easily spectrophotometrically. It is obvious that a more simple tetraën would be better suited for our study, but these compounds are rare, are generally very labile, and usually are too insoluble in water. Pimaricin, on the other hand, has several advantages: it is commercially available, it is relatively stable, and the solubility is sufficient for the experimental conditions.

15a. B. Hendriks and W. Berends, Rec.trav.chim., 77, 145 (1958).

16. A.P. Struyk, I. Hoette, C. Drost, J.M. Waisvisz, Th. van Eek and J.C. Hoogerheide, Antibot.Ann., 878 (1957, 1958).

17. J.B. Patrick, R.P. Williams, C.F. de Wolf and J.S. Webb, J.Am.Chem.Soc., 80, 6688 (1958).

18. J.B. Patrick, R.P. Williams and J.S. Webb, J.Am.Chem.Soc., 80, 6689 (1958).

Pimaricin has maxima at 281, 292, 304 and 319 mμ. The spectrum of riboflavin is more complicated, with maxima at 223, 268, 373 and 445 mμ (fig. 3).

Several photodynamic destructions in which riboflavin is involved have been described in the literature, e.g., aerobic photooxidation of phenols, ascorbic acid, 3-indoleacetic acid, and histidine[19, 20]; usually an oxidation of the substrate or a free radical mechanism is supposed to occur.[21-24]

From earlier experiments and those to be described in this paper it will appear that neither is acceptable as an explanation for our observations of the photodynamic behavior of an aqueous solution of the system riboflavin-pimaricin.[25]

As a source of irradiation we used a Kromayer high-pressure mercury lamp. This lamp was placed at a distance of 3 cm. from the aqueous solution. By using an interference filter light with a wave length of about 443 mμ was obtained. The destruction of pimaricin could be followed by measuring the absorbancies at 304 and 319 mμ by means of a Unicam spectrophotometer SP 500. About 23% of the pimaricin was destroyed after 5 min. of irradiation. The absorption spectrum of riboflavin remained unchanged under these conditions and no pimaricin destruction could be observed by irradiating pimaricin alone.

---

19. P.A. Kolensnikow, Biokhimiya, 22, 434 (1958).

20. A.W. Galston, Science, 111, 619 (1950).

21. H.R. Merkel and W.J. Nickerson, Biochim.Biophys.Acta, 24, 115 (1957).

22. W.J. Rutter, Acta Chem.Scand., 12, 438 (1958).

23. L.P. Vernon and E.D. Ihnen, Biochim.Biophys.Acta, 24, 115 (1957).

24. L.P. Vernon, Biochim.Biophys.Acta, 36, 117 (1959).

25. E. Zondag, J. Posthuma and W. Berends, Biochim.Biophys.Acta, 39, 178 (1960);
J. Posthuma and W. Berends, Biochim.Biophys.Acta, 41, 538 (1960); 51, 392 (1961).

Though riboflavin ia decomposed by prolonged irradiation, yielding lumichrome, a free radical mechanism is not likely to occur in the reaction just described because lumichrome alone is also able to bring about this pimaricin destruction. (Lumichrome is stable to the light used in these experiments (368 and 395 mμ) and even to ultraviolet light of shorter wave lengths.[26]). Moreover, the photolysis of riboflavin in alcohol is much stronger than in water and nevertheless no destruction of pimaricin takes place in alcohol.

Vernon[23, 24] has suggested a free radical mechanism in which excited riboflavin decomposes water; the OH-radicals thus formed oxidize the substrate, or give rise to H_2O_2 in the absence of a substrate. We have strong evidence, however, that no H_2O_2 is formed in irradiated aqueous solutions of lumichrome.

Lumichrome is completely stable to ultraviolet radiation[26], but addition of a very small amount of H_2O_2 (10^{-6}M) brings about a very rapid destruction of lumichrome. Consequently any H_2O_2 formed under the experimental conditions would easily be detected. As apparently no H_2O_2 arises the formation of OH.-radicals is also not very probable.

If oxidation of pimaricin, accompanied by the simultaneous reduction of riboflavin, were the primary cause of the reaction between riboflavin and pimaricin it could be expected that oxygen would accelerate the photodynamic reaction. However, enhancement of the oxygen concentration in solution diminished the destruction of pimaricin.

Bubbling through N_2, prior to illumination, on the other hand strongly accelerates the pimaricin destruction. The remarkable inhibiting effect of oxygen on the photodynamic destruction in the systems riboflavin-pimaricin and lumichrome-pimaricin suggests a direct transfer of excitation energy and makes it unlikely that free radicals are involved. It is not probable that this transfer proceeds via a riboflavin-pimaricin or lumichrome-pimaricin complex because we were not able to detect such complexes by absorption and fluorescence measurements.

---

26. General Electric Germicidal lamp (4-watt).

Szent-Györgyi[27] has recently given some examples of charge-transfer complexes, but the concentrations used in his experiments (10^{-3} M) were much higher than in ours ($< 10^{-5}$ M). However, we cannot exclude completely intermediate complex formation.

Further observations of the reaction mentioned have brought us to the conclusion that the sensitizers riboflavin and lumichrome react in their triplet states. These triplet states have a rather long lifetime compared with the corresponding singlets and their importance in many photosensitized reactions in non-aqueous solutions has been recognized in the last decade.[28, 29] These long-lived excited states can be demonstrated by phosphorescence of the frozen solutions.

In our reactions the average time for collisions between riboflavin and pimaricin in a 10^{-5} M solution will be about 10^{-4} - 10^{-5} sec.[30] From the life-time of the singlet state, about 10^{-8} sec., it is evident that only relatively long-lived excited states like triplets have a reasonable chance to transfer their energy.

We therefore propose the following reaction sequence as an explanation of the photodynamic destruction of pimaricin

$$\text{Riboflavin} + h\nu \longrightarrow \text{Riboflavin}^* \text{ (singlet)} \longrightarrow \text{Riboflavin}^* \text{ (triplet)} \qquad (1)$$

$$\text{Riboflavin}^* \text{ (triplet)} + \text{Pimaricin} \longrightarrow \text{Riboflavin}^* + \text{Pimaricin}^* \text{ (triplet)} \qquad (2)$$

In reaction 1 riboflavin is first excited by a 445 mμ light quantum to its singlet state and next a singlet-triplet transition takes place (conversion of the spin moment). From this state an energy transfer to a pimaricin is possible if the triplet of pimaricin is lower than the similar state of riboflavin.

---

27. I. Isenberg and A. Szent-Györgyi, Proc.Natl.Acad.Sci.U.S., 44, 857 (1958); 1231 (1959). G. Karreman, I. Isenberg and A. Szent-Györgyi, Science, 130, 1191 (1959).

28. C. Reid, "Excited States in Chemistry and Biology", Butterworths Scientific Publications, London, 1957.

29. G. Porter, Proc.Chem.Soc. (London), 291 (1959).

30. G. Oster and A.H. Adelman, J.Am.Chem.Soc., 78, 913 (1956).

Reaction 2 is allowed by spin-conservation rules.[31] A few examples of such transfers have been described recently.[29, 32]

The over-all result of this triplet-triplet transfer would be an excited pimaricin molecule. It is conceivable that a very sensitive compound like pimaricin with its reactive tetraën structure decomposes if it is excited. The lability of pimaricin is demonstrated by its sensitivity to ultraviolet light.

This reaction mechanism is supported by the effect of several substances upon the system riboflavin-pimaricin. A compound that quenches the phosphorescence of riboflavin is supposed to act in this way by facilitating the radiationless dissipation of excitation energy by collisions with molecules of the solvent. Consequently, the lifetime of the triplet state is shorter. This means the triplet-triplet transfer is less probable.

It appears that the phosphorescence quenching effect of the compounds tested ran completely parallel to their degree of inhibition of the photodynamic destruction of pimaricin in the combination riboflavin-pimaricin.

Table III.

Effect of Various Substances on Photodynamic Destruction of Pimaricin, and on Riboflavin Phosphorescence.

	Photodynamic destruction of pimaricin		Riboflavin phosphorescence	
Added substance	Conc.,M	% loss pimaricin	Concn.,M	Color of frozen part of the soln.
...	...	23	...	Orange
Cystine	10^{-3}	35	10^{-3}	Orange
Cysteine	10^{-3}	33	10^{-3}	Orange
Methionine	10^{-3}	21	10^{-3}	Orange
Thioglycolic acid	10^{-3}	79	10^{-3}	Yellow
Glutathione	10^{-3}	58	10^{-3}	Yellow
Na_2SO_3	10^{-3}	93	10^{-3}	Orange
Ethyl iodide	10^{-3}	22	10^{-3}	Orange
Monoiodoacetic acid	10^{-3}	23	10^{-3}	Orange
KI	10^{-3}	2	10^{-3}	No color
KI	10^{-4}	5	10^{-4}	No color
Potassium rhodanide	10^{-3}	5	10^{-3}	No color
Thiourea	10^{-3}	5	10^{-3}	No color
Thiouracil	10^{-4}	8	10^{-4}	No color
Ascorbic acid	$5x10^{-5}$	Inhibition	$5x10^{-5}$	No color
Hydroquinone	$5x10^{-5}$		$5x10^{-6}$	No color
Methanol	75 vol.%	2	5 vol.%	Yellow
Ethanol	96 vol.%	0	5 vol.%	Yellow
Pimaricin*	..	...	$2x10^{-5}$	Weakly orange

* Because of the low solubility of the pimaricin a $2x10^{-6}$ M solution of riboflavin was used to observe the phosphorescence quenching.

Table IV.

Aerobic conditions, light acceptor riboflavin (1.9×10^{-5} M)

Salt added		Concn. of the salt (mM)	% decrease of extinction of pimaricin	Color of the frozen soln.
	...	...	54	Orange phosphor.
Paramagnetic	$NiCl_2$	2.5	42	Colorless
	$CuCl_2$	2.5	24	Colorless
Paramagnetic	$MnCl_2$	2.5	45	Colorless
	$CoCl_2$	2.5	43	Colorless
	$CrCl_3$	2.5	43	Colorless
Diamagnetic	$MgCl_2$	2.5	53	Orange phosphor.
	$ZnCl_2$	2.5	51	Orange phosphor.
	$CaCl_2$	2.5	52	Orange phosphor.
	$CdCl_2$	2.5	52	Orange phosphor.
	KCl	2.5	52	Orange phosphor.
	NaCl	2.5	52	Orange phosphor.

Anaerobic conditions, light acceptor lumichrome (2×10^{-6} M)

Salt added		Concn. of the salt (mM)	% decrease of extinction of pimaricin	Color of the frozen soln.
	...	...	22	Yellow phosphor.
Paramagnetic	$NiCl_2$	0.5	17	Colorless
	$CuCl_2$	.5	11	Colorless
Paramagnetic	$MnCl_2$	.5	20	Colorless
	$CoCl_2$	.5	15	Colorless
	$CrCl_3$	.5	18	Colorless
Diamagnetic	$MgCl_2$	.5	21	Yellow phosphor.
	$ZnCl_2$	.5	21	Yellow phosphor.
	$CaCl_2$	.5	20	Yellow phosphor.
	$CdCl_2$	.5	21	Yellow phosphor.
	KCl	.5	21	Yellow phosphor.
	NaCl	.5	21	Yellow phosphor.

On the other hand, compounds without visible observable quenching effect show no inhibition but in many cases even accelerate the pimaricin destruction.

---

31. G. Porter and M.R. Wright, "Symposia Faraday Soc., Nottingham", 1959.

32. A. Terenin and V. Ermolaev, Trans.Faraday Soc., 52, 1042 (1956).

The quenching effect of compounds on the phosphorescence of riboflavin was tested by adding them to a 2×10^{-5} M aqueous solution or riboflavin and visually observing the phosphorescence in ultra-violet light after freezing.[33]

In order to keep the solutions at constant pH a phosphate buffer (0.06 M, pH 6.8) was used. The concentration of riboflavin was 2×10^{-5} M, that of pimaricin 9×10^{-6} M.

The ultraviolet light was obtained from a Philips lamp H.P.W. 125. The results are given in Table III.

Besides the compounds mentioned in this table, the paramagnetic metal ions are particularly active as quenchers of phosphorescence, which means that these ions shorten the triplet lifetime. For this reason we have tested a number of para- and diamagnetic ions in the same manner as we did the compounds of Table III.

The aqueous solutions containing 9×10^{-6} M pimaricin and 0.01 M sodium acetate-acetic acid (pH 4.3) were added riboflavin or lumichrome and the salts recorded in Table IV. The solutions were exposed to light in glass tubes (inner diameter 2.2 cm) for 5 min. Again the light was obtained from the Kromayer lamp and the desired wave length was isolated by means of a Schott interference filter. For the system riboflavin-pimaricin light of 443 mμ was used. In the case of lumichrome-pimaricin a wave length of 395 mμ was chosen. In the anaerobic experiments the air was removed by bubbling through nitrogen during 20 min. prior to illumination. Riboflavin could not be used under anaerobic conditions because an appreciable destruction of this compound occurs in the time of irradiation.

Table IV shows that all paramagnetic ions tested diminish the photodynamic pimaricin decomposition and that the phosphorescence of riboflavin and lumichrome also is quenched by these ions. Diamagnetic ions have no effect at all. These observations apparently are in complete agreement with the paramagnetic quenching of the triplet state. These ions affect only the triplet

---

33. A. Szent-Györgyi, "Bioenergetics", Academic Press, New York, N.Y., 1957.

state of the sensitizer, for the direct destruction of pimaricin by ultraviolet light is not influenced by the paramagnetic compounds.

For the study of the triplet states of riboflavin and lumichrome, pimaricin was not quite satisfactory because it has a very complicated structure and consequently it is not known exactly what kind of conversion is responsible for the decrease of extinction. We therefore looked for a more simple compound and we found the possibility of replacing pimaricin by stilbene derivatives.

The first compounds tested were cis- and trans-stilbene carboxylic acid-4. The cis compound shows absorption maxima at 230 and 288 mμ; those of the trans compound are found at 230 and 317 mμ with a shoulder at 308 mμ (fig. 4).

Irradiation of either the cis or the trans form in the presence of lumichrome rapidly leads to a spectrum which in the region above 250 mμ agrees with a mixture of about 40% cis and 50% trans of the original concentration (fig. 4). This equilibrium mixture slowly decomposes on further irradiation (fig. 5).

A similar equilibrium mixture is obtained by irradiation in the absence of the sensitizer with light of 253.7 mμ. Apparently a cis-trans and a trans-cis rearrangement are involved. Preliminary experiments have shown that the lumichrome-sensitized isomerizations also are inhibited by neutral quenchers like O_2, KI and KCNS, just as is the destruction of pimaricin in the system lumichrome-pimaricin, so we may assume that in these photosensitized rearrangements a triplet-triplet transfer is involved.

Investigations with stilbene sulfonic acids instead of the carboxylic acids are in progress. The quantum efficiencies of the reactions also are being studied at present.

To get a more quantitative idea of the efficiency of the energy transfer an actinometric method of determining the quantum yield[34] was used for the systems lumichrome-cis stilbene carboxylic acid.4 and lumichrome-pimaricine. The essential

---

34. C.G. Hatchard and C.A. Parker, Proc.Roy Soc. A 235 (1956) 518.

reaction used in this method is the photolysis of potassium-ferri oxalate by light of a wavelength shorter than 490 mμ. The following reaction takes place:

$$2\left[Fe(C_2O_4)_3\right]''' \xrightarrow{h\nu} 2\,Fe(C_2O_4) + 3\,C_2O_4'' + 2\,CO_2$$

By making the p_H of the solution strongly acid (about p_H 1) the ferro oxalate dissociates according to:

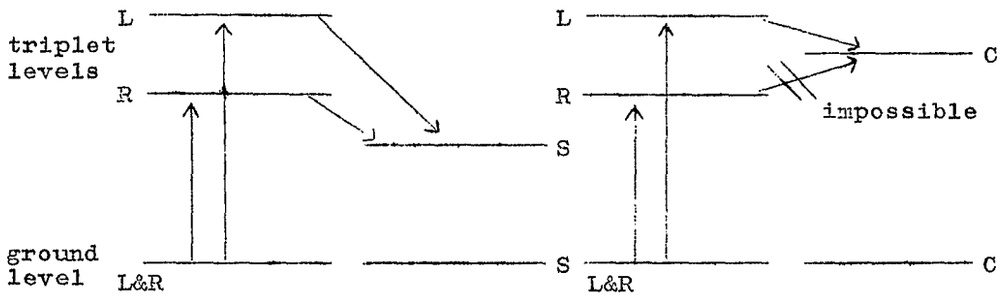
$$Fe(C_2O_4) \longrightarrow Fe^{\cdot\cdot} + C_2O_4''$$

Next the ferro ions formed in this reaction are determined by complexformation with 1,10 phenanthroline at p_H 3,5. The extinction of the red complex, $Fe(Phen)_3^{\cdot\cdot}$, is measured in a spectrophotometer at 510 mμ. At p_H 3.5 there is no ferro left free in solution and therefore the amount of ferro produced by the radiation is known. Subsequently the intensity of the light-source can be calculated with the quantum efficiency determined by Hatchard and Parker[34] for this reaction. The same light intensity has been used for the irradiation of an aqueous solution of lumichrome and cis-stilbene carboxylic acid-4. Light of a wavelength 406 mμ was selected from a Philips HP 125 mercury lamp by a Schott interference filter. Care was taken that all the incident light was absorbed during the reaction by making the light path 10 cm in length. The reaction studied is the isomerization of cis-stilbene carboxylic acid-4 to the trans isomer by lumichrome. The amount of the trans-isomeer formed can be determined by the rise in extinction at 317 mμ (absorption maximum). The mean quantum efficiency during the first 5 minutes of the irradiation turned out to be 0.035 for the energy transfer between lumichrome and cis-stilbene carboxylic acid-4.

A quantum efficiency in the same order of magnitude has been found for the system lumichrome-pimaricine.

Another system probably dealing with a cis-trans isomerization is lumichrome- cis-cinnamic acid. This is again performed in aqueous solution. Cis-cinnamic acid has absorption-maxima at 205 and 254 mμ. During the irradiation the latter becomes higher and shifts towards 272 mμ. A remarkable finding has been the difference between lumichrome and riboflavin for

this system. No sensitization occurs when riboflavin is used instead of lumichrome. The stilbene derivatives are isomerized both in the presence of riboflavin and of lumichrome. It seems very likely that the triplet level of cis-cinnamic acid is somewhat higher than that of riboflavin but below or at the same level as that of lumichrome.



L: lumichrome
R: riboflavin
S: cis-stilbene sulfonic acid-4
C: cis-cinnamic acid

The dependence of the energy transfer on the p_H has been measured for the system lumichrome- cis stilbenesulphonic acid-4 between p_H 3,6 and p_H 7. The velocity of the conversion of the stilbene derivative decreases by changing the p_H to lower values (p_H 7 ⟶ p_H 3,6). At still lower p_H the extinction at 312 mμ does not rise any more, but decreases during the irradiation in the presence of lumichrome. This means that the formation of the trans isomer (maximum at 312 mμ is hampered or that another reaction takes place.

To get an opinion of the properties of lumichrome in the triplet state, assumed to be the active excited form in our systems, we have made a study of:

a. phosphorescence in the solid state,

b. triplet-triplet absorption in water at room temperature.

a. It is possible to determine the triplet levels of the substances participating in the reaction by a close examination of the light of phosphorescence. In the first place we have been looking for the origin of the orange red emission of riboflavin in frozen solution. According to Szent-Györgyi[33] this would be

caused by phosphorescence. However, we could not detect any phosphorescence of riboflavin (or lumichrome) with our phosphorescope under the experimental conditions. With this instrument phosphorescence with a lifetime of 5 milliseconds or longer could be detected.

In figure 6 the spectrum isngiven for riboflavin in aqueous solution and in ice. These spectra are measured with a Hilger-Watts quartz-spectrograph and Ilford HP 3 plates. The solutions have been illuminated with a U.V. lamp (Philips HPW 125). The exposure time varies between 3/4 and 1 1/2 hour. It appears that on freezing the solution of the riboflavin a shift of the emission spectrum takes place to the red. The life-time, however, remains short, what may lead one to conclude that we are not dealing with phosphorescence.

This is in accordance with the findings of Dhéré and Castelli[35]. From the results of their experiments they suggested the emission of riboflavin and some other dyes in ice to be a fluorescence of a dimer or polymer of these substances. These conclusions have been confirmed lately by Morozov et al.[36]

To study the real phosphorescence we must change to other solvents. Most of the determinations have been performed in a glycerol-water mixture (1:1) because on cooling a glass is obtained. Only lumichrome has been measured in pyridine-glycerol (higher solubility). The same positioning as for the measurement of the fluorescence has been used. The only difference consists in the insertion of a phosphorescope between the solution and the recording system. The temperature during the measurements has been between 100° and 180° degrees centigrade below freezing by occasionally cooling of the tube with the solution in liquid nitrogen. The phosphorescence spectra obtained are given in figure 7.

---

35. Ch. Dhéré, V. Castelli, Compt.Rend. 206 (1938) 2003.

36. L.A. Tumerman, Y.V. Morozov, Y.I. Naberukhin, Biofysika 6 (1961) 556.
Y.V. Morozov, Y.I. Naberukhin, G.V. Gursky, Optica e Spectroscopya 12 (1962) 599.

It should be possible to determine the triplet levels of the energy accepting substances (e.g. pimaricine and atilbeme derivatives) by measuring those of the sensitisizing dyes in the way, just described. Only when the level of the dye is higher or equal to that of the acceptor a transfer of energy, resulting in a detectable chemical change, is possible. This is under study.

b. The most direct method to detect a long lived excited state in solution of riboflavin or lumichrome is by way of the "flash photolysis" method. These experiments have been performed during a stay at the laboratory of prof. G. Porter in Sheffield (England). For this work two different apparatus have been used:

the "flash photographic apparatus"

the "flash photoelectric apparatus". (see fig. 8).

With the first technique the whole absorption spectrum is measured of the compound under study, immediately after the solution has been subjected to a flash with a very high energy (250-1000 Joule) and short duration ($< 50\ \mu$ sec.). This is done by flashing the solution with a second flash of lower energy (50 Joule, spectroscopic flash) and measuring the light transmitted by a spectrograph. The length of the cell is about 20 cm. In this way the complete absorptionspectrum of the excited state with long lifetime can be recorded at a certain time after the big flash and, by varying the interval between the two flashes the return to the ground state can be made visible. This transition of the excited molecules from the triplet state to the ground state can be studied more accurately with the second method (flash photoelectric method). Now the change in the absorptionspectrum following the big flash is measured constantly. The light of a stationary light source passes the cuvet, is led through a monochromator and falls on a photoelectric cell. The electric signal is put on the vertical plates of an oscilloscope. During the photolysis flash the amount of light transmitted changes suddenly and returns relatively slowly to the original value (transmission of the ground state of the molecules). The decay curve on the screen of the oscilloscope can be photographed and analyzed. Accurate measurements are possible from about 50 µsec. after the photolysis flash.

With the first method the absorptionspectrum has been measured of the long lived excited state of lumichrome. The new absorptionband we have mentioned before turns out to consist of at least two absorptionmaxima with a partly overlap and a different lifetime. Apart from this change in the spectrum between 425 and 650 mμ a strong change takes place in the ultraviolet part of the spectrum during the flash.

At two different wavelengths, 480 and 570 mμ, the decay of the long lived excited state has been measured with the second technique. The two curves differ a great deal, at 480 mμ the component with the longest lifetime dominates, at 570 mμ that with the shortest lifetime. It has not been possible to get a complete separation of both components by choosing a special wavelength. Trials to obtain the total curve by combining two first order curves have not been successful either (fig. 9).

Subsequently the influence of known triplet quenchers on the excited states detected has been studied for lumichrome. Addition of KI or paramagnetic ions results in a diminishing of the amount of the component with the longest lifetime, without shortening this lifetime in a measurable way. The amount of the component with the short lifetime under these conditions becomes so small that the lifetime cannot be measured any more. All experiments have been performed in the complete absence of oxygen. This gas, being paramagnetic, namely has the same effect of the other quenchers: disappearance of the component with the short lifetime and decreasing the amount of the other component. A complete removal of oxygen was obtained by cooling the cuvet with the solution in ice, evacuating the cuvet by means of a vacuum line, warming up and shaking and repeating this procedure 4 or 5 times. The decay-curve in the presence of oxygen again is neither pure first nor second order.

The amount of both components is also diminished by addition of trans stilbene carboxylic acid-4. This substance also seems to reduce the lifetime of the component with the short lifetime.

These experiments do not give clear proof that the energy absorbed by lumichrome is transferred via a triplet-triplet

transfer to the stilbene derivative. Nevertheless it seems very likely that one of the components detected is lumichrome in the triplet state, very probably that with the short lifetime.

III. Other investigations.

1. Irradiation of dyes with visible light.

It is only possible to make a fruitful study of a photosensitization by having enough knowledge of the effect of the light used on the dye itself. Though substances like nucleic acids may influence the reaction, one cannot do without these controls. In this connection we may point to the difference and the resemblance in the behaviour of riboflavin and lumichrome (see the theoretical study on photosensitization in this and last year's report).

Usually the dyes known to be photosensitizing are rapidly broken down in solution by visible light. This holds for acridin orange, proflavin, riboflavin, neutral red and safranine. There are however a few stable dyes; we have found lumichrome, berberine and 8-methoxy-psoralen. To demonstrate a photosensitization an isolation of the substance under investigation (e.g. nucleic acid) from the dye is necessary after the irradiation (as has been done with soluble ribonucleic acid p. 8) or one should use a stable dye. In this case one must take care that the change of the environmental conditions may lead to a destruction of the dye. An irradiation of 8-methoxypsoralen in frozen solution, e.g., gives rise to the formation of a new compound (probably the dimer of the psoralen). This substance misses the characteristic absorption of the original dye and the reaction can be reversed by irradiation of the liquid solution with U.V. light.

2. Preparation of polynucleotides with known composition.

To study the effect of radiation on polynucleotides we have available only the two types of nucleic acid, deoxyribonucleic acid and ribonucleic acid. Though these substances are very interesting with respect to their biological value, they have the

drawback that the sequence of the nucleotides in these polymers is rather arbitrary. Since we have learned from the dimerization of two neighbouring thymine molecules that it is very important which molecules are next to each other, the preparation of polynucleotides containing only one base or having a regular construction may be considered very important. Lately the group of Shugar has studied the effect of U.V.-radiation on oligo- and poly-thymidylic acid.[37] The results agree with those obtained by us with a sample of tetrathymidylic acid, a gift of prof. R.L. Sinsheimer (figure 10). Again we see a partial conversion of the thymine molecules to a dimer (maximal 35%). We probably are dealing with an equilibrium as in the case of thymine, uracil and orotic acid.

Schachman and collaborators discovered merely accidently the formation of a very special polynucleotide upon mixing deoxy-adenosinetriphosphate and thymidinetriphosphate with the Kornberg enzym polymerase and the necessary additions but without a priming nucleic acid. After a lag time of a few hours suddenly a polynucleotide is formed. On investigation it appeared to be composed of adenine and thymine nucleotides in alternating sequence.[38]

To obtain a large amount of this special polynucleotide, we started an isolation of the Kornberg enzym from Escherichia coli. Though the recipe[39] is not very easy to work with as a result of the difficulties in testing, we have succeeded in isolating a fairly active preparation. We try to improve the testing in different ways, mostly physicochemically, to get a better control during the isolation. As soon as we have obtained a sufficient amount of the enzym we will start with the preparation of polyAT according to Schachman.

Another method to prepare polynucleotides has been described by Schramm et al.[40]. The polymers are obtained purely chemically

---

37. E. Satumpf, D. Shugar, Bioch.Biophys.Acta, 61 (1962) 555.
38. H.K. Schachman, J. Adler, C.M. Radding, I.R. Lehman, A. Kornberg, J.Biol.Chem., 235 (1960) 3242.
39. I.R. Lehman, M.J. Bessman, E.S. Simms, A. Kornberg, J.Biol.Chem., 233 (1958) 163.
40. G. Schramm, H. Grötsch, W. Pollmann, Angew.Chem. 74 (1962) 53.

by condensation of the mononucleotides with polyphosphoric ester. In this way we have already prepared a high molecular polyadenylic acid. This reaction seems to depend on the presence of special priming polymers. This will be investigated in the next future.

Summary.

In order to study the effect of radiation (visible light in the presence of special dyes and U.V.) on nucleic acids in more detail, we turned to the use of biologically active and of ^{14}C labeled preparations. The former products enabled us to detect an inactivation of DNA by visible light with presence of neutral red, methylene blue, fluorescein and lumichrome. The dye last mentioned has been preferred in a further study on account of its stability to the light used. The chemical background of the photosensitization of DNA is a destruction of a part of the guanine as has been demonstrated with DNA, containing 8-^{14}C guanine. The presence of oxygen is obligatory for the reaction and a development of $^{14}CO_2$ occurs. Still more $^{14}CO_2$ is liberated during the hydrolysis of the nucleic acid with perchloric acid. The combination of visible light and lumichrome is also able to convert guanine and its nucleotides to substances lacking the characteristic absorption of this purine. The conversion is slowed down if paramagnetic metal ions are present, suggesting a reaction on a triplet-level. By chromatography an isolation of the photoproduct is underway.

The photodecomposition of the polyene fungicide pimaricin sensitized by riboflavin and lumichrome in aqueous solution has been found. The inhibiting effect of added substances including paramagnetic ions on this destruction runs completely parallel to their quenching effect on the phosphorescence of the sensitizers. A triplet-triplet transfer is therefore proposed as a mechanism for this destruction. This mechanism is probably also involved in the photosensitized cis-trans and trans-cis rearrangements of stilbene compounds in aqueous solutions in the presence of lumichrome. Evidence for this mechanism has also been found from flash photolysis and phosphorescence experiments. At least two

long lived excited species of lumichrome have been detected. One of them is completely quenched by paramagnetic ions and by the stilbene derivative. An overall quantum yield of 0.035 has been found for the energy transfer in this system.

The ability of soluble ribonucleic acid to bind amino acids appeared to be decreased by visible light in the presence of berberine and by U.V. irradiation. No explanation of this phenomenon on the molecular level can be given thus far.

The preparation of synthetic polynucleotides of known composition by enzymatic and organic-chemical methods is under study.

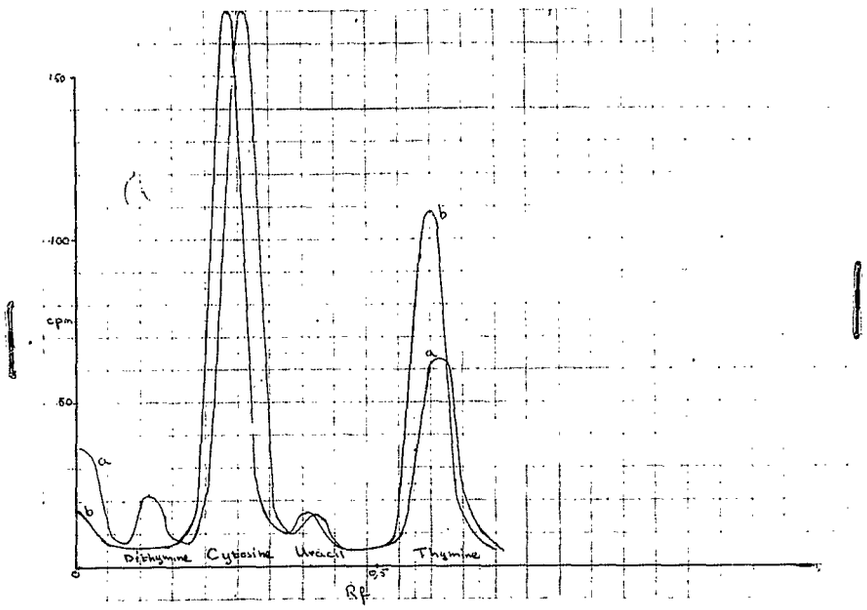


Fig. 1. Paperchromatogram of a DNA hydrolysate. Irradiation of the DNA solution (1 mg in 5 ml water) was performed with a General Electric Germicidal Lamp (4 W.) during 15 minutes at a distance of 5 cm.
Solvent: butanol-NH_3
Curve a: DNA irradiated
Curbe b: DNA not irradiated

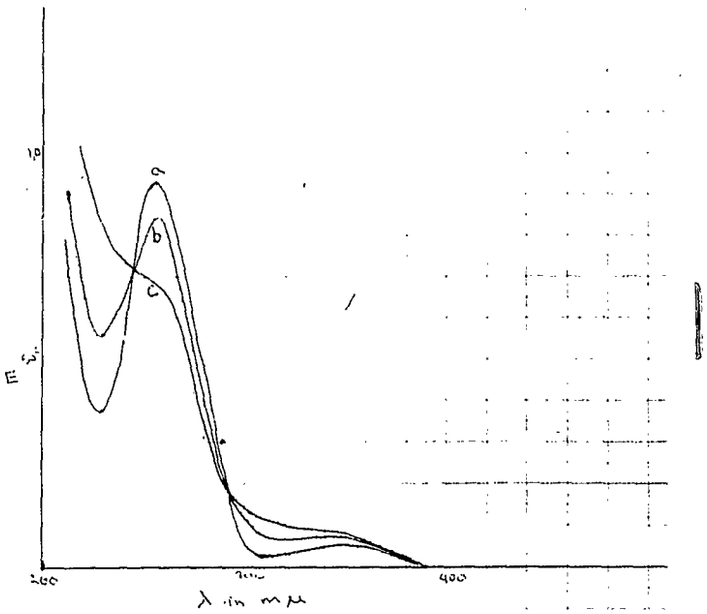


Fig. 2. Spectral changes of deoxyguanylic acid (0,4 μM) upon irradiation with a Philips HP 125 light source in the presence of lumichrome (0,03 μM) at a distance of 20 cm (sum spectra given).
Curve a: prior to irradiation
Curve b: after 10 minutes of irradiation
Curve c: after 120 minutes of irradiation

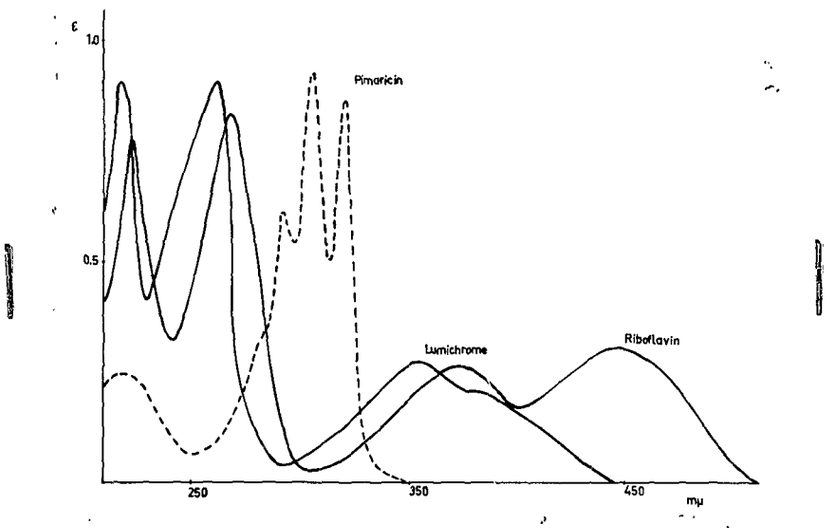


Fig. 3. Absorption spectra of pimaricin ($1.44.10^{-5}$ M), riboflavin ($2.66.10^{-5}$ M) and lumichrome ($2.24.10^{-5}$ M), Light path 1 cm.

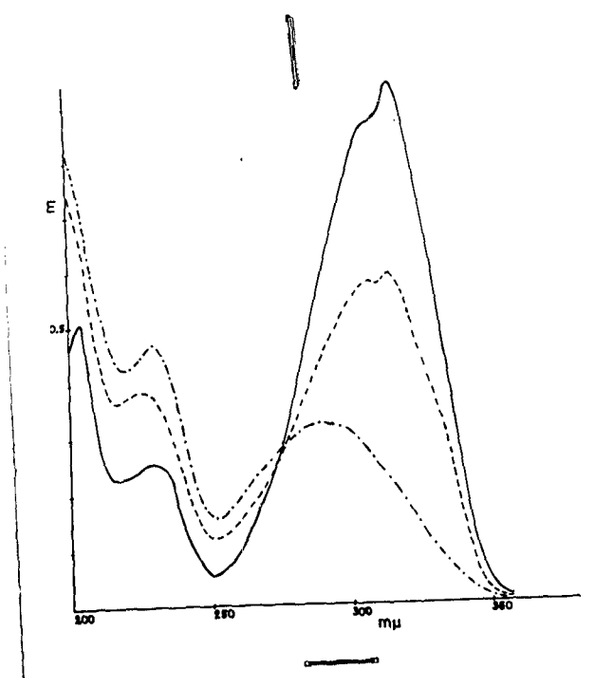


Fig. 4. Irradiation of aqueous solutions of $2.4.10^{-5}$ M cis and trans stilbene carboxylic acid-4 in the presence of $3.7.10^{-5}$ M lumichrome.

-.-.-.-. cis stilbene carboxylic acid-4

———— trans stilbene carboxylic acid-4

-------- after irradiation sensitized by lumichrome

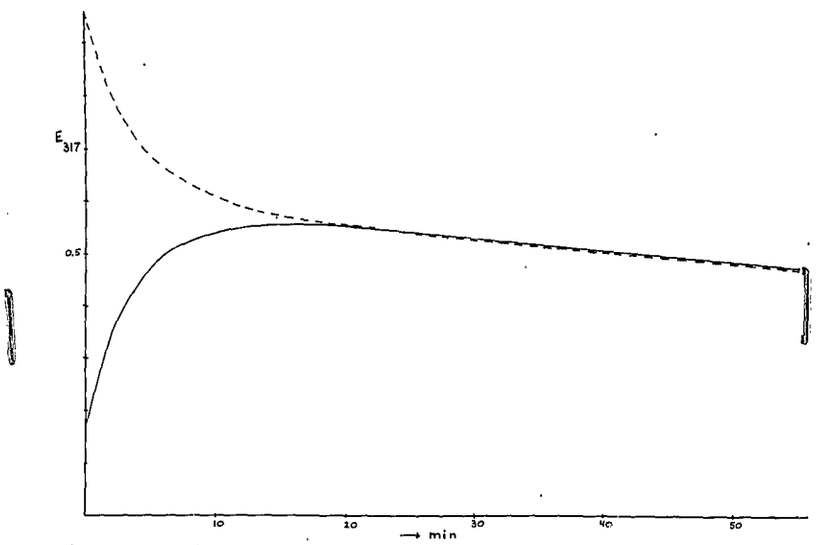


Fig. 5. Change of the extinction at 317 mμ (maximum of the trans compound) during the irradiation in the presence of lumichrome.

———— cis stilbene carboxylic acid-4

---------- trans stilbene carboxylic acid-4

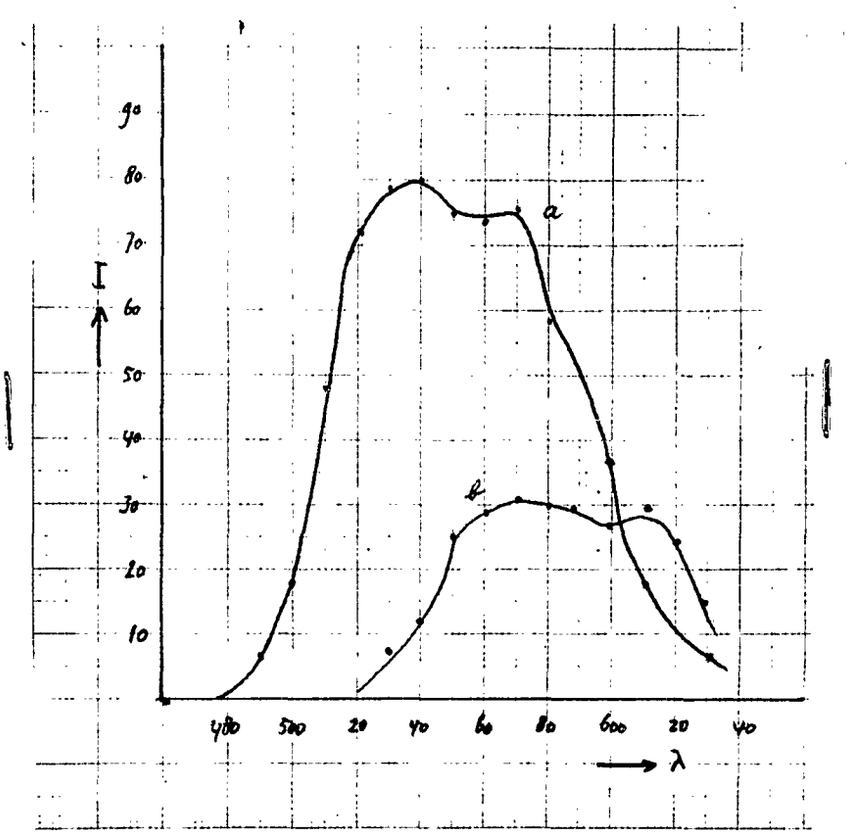


Fig. 6. Fluorescence spectra of riboflavin (10^{-4} M).
a. In aqueous solution.
b. In ice.

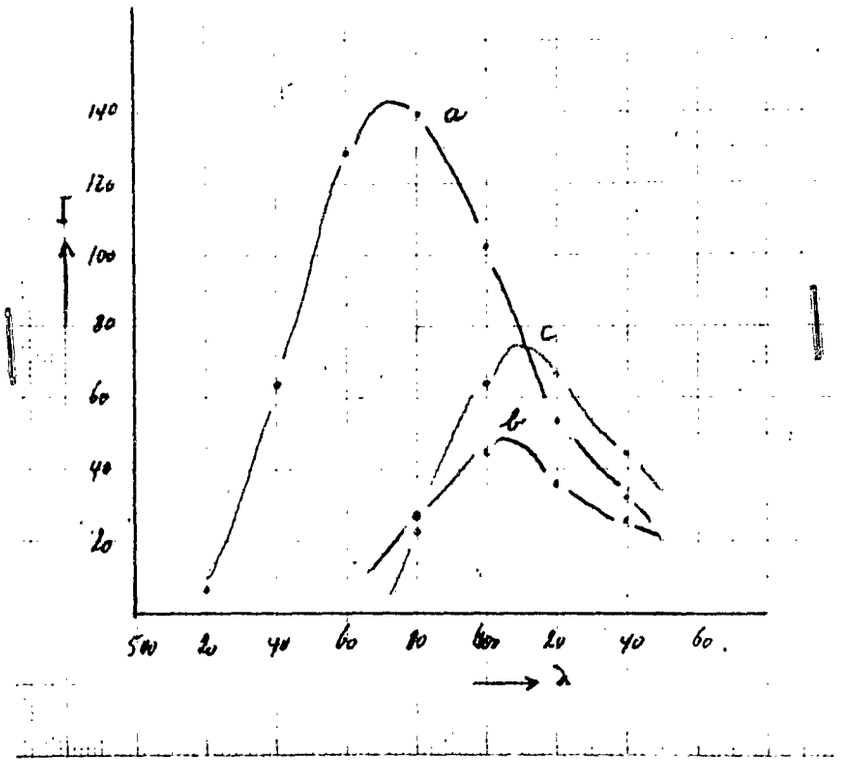


Fig. 7. Phosphorescence spectra recorded at temperatures between -180 and -100° C.

a. 10^{-3}M lumichrome in glycerol-pyridine 1:1

b. 2.10^{-4}M riboflavin in glycerol-water 1:1

c. 5.10^{-3}M acridine orange in glycerol-water 1:1

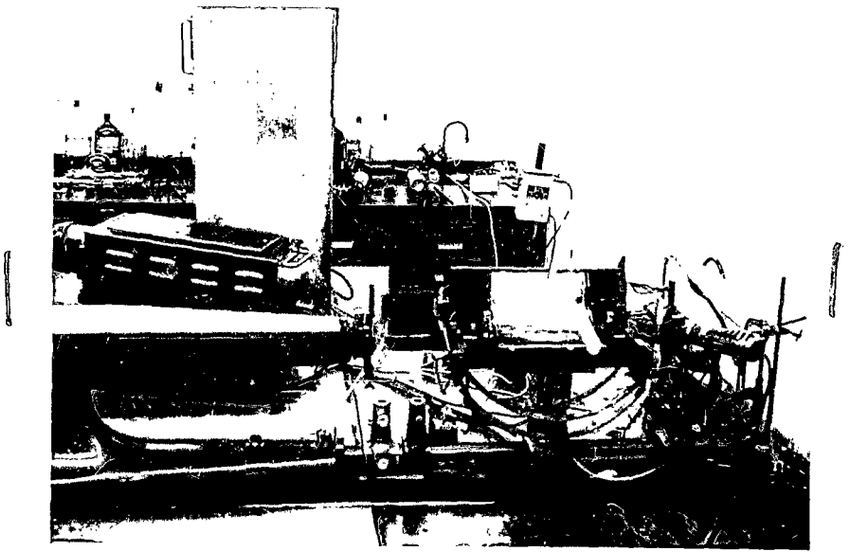


Fig. 8. The flash-photographic apparatus.

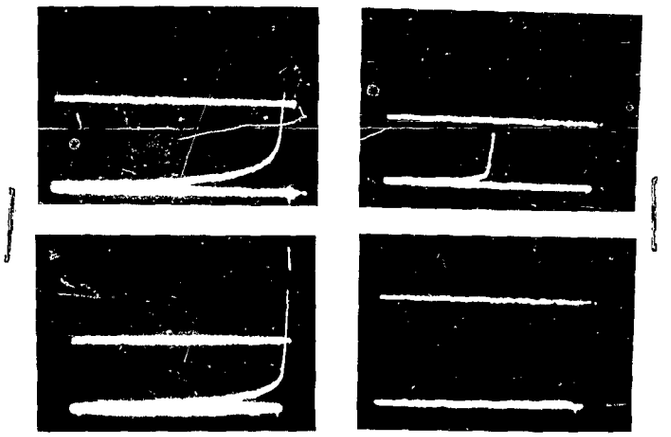


Fig. 9. Some oscillograph traces of the transmittance at 570 mμ of an aqueous lumichrome solution against time (from right to left).
The pictures were taken immediately after a 1000 Joules photolysis flash, filtered by a 1 cm cupper nitrate solution (saturated).

top left: degassed aqueous solution of lumichrome ($5.8.10^{-6}$M)

bottom left: 2.10^{-3}M $CaCl_2$ added prior to degassing

top right: 2.10^{-4}M $MnCl_2$ added prior to degassing

bottom right: 2.10^{-3}M $NiCl_2$ added prior to degassing

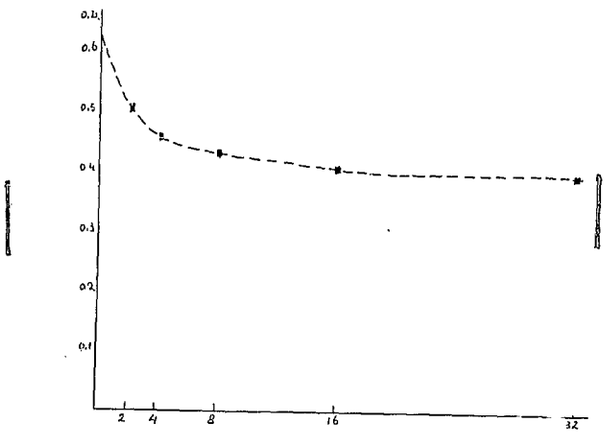


Fig. 10. Decrease in extinction (O.D.) of a solution of tetrathymidylic acid upon irradiation with a U.V. light source. Time given in minutes.